PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 16 June 2000 (16.06.00)	
International application No. PCT/EP99/07911	**Applicant's or agent's file reference** NO 5650/WO
International filing date (day/month/year) 15 October 1999 (15.10.99)	**Priority date** (day/month/year) 20 October 1998 (20.10.98)
Applicant VIDAL, Karine et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

08 May 2000 (08.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
NO 5650/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/07911	15/10/1999	20/10/1998
Applicant		
SOCIETE DES PRODUITS NESTLE S.A. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   PROTEIN FOR TREATMENT OR PREVENTION OF A GASTROINTESTINAL TRACT DISORDER

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/EP 99/ 07911

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18 and 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]P 99/07911

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 A23L1/305 A23J1/20 A61P1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A23L A23J A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 99 61468 A (GEMMA BIOTECHNOLOGY LTD ;FILIPP DOMINIK (CA); JULIUS MICHAEL H (CA) 2 December 1999 (1999-12-02) the whole document	1-5,7-19
X	WO 98 22580 A (ALIZADEH KHIAVI K ;FILIPP DOMINIK (CA); JULIUS MICHAEL H (CA); WEL) 28 May 1998 (1998-05-28) the whole document	1-5, 7-10, 12-15,18
X	WO 92 04908 A (IMTOX PRIVATINSTITUT FUER IMMU) 2 April 1992 (1992-04-02) the whole document	8-10,13, 14

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2000	09/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Panzica, G

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WANG Y ET AL: "DETECTION AND IDENTIFICATION OF SOLUBLE CD14 IN BOVINE MILK" MOLECULAR BIOLOGY OF THE CELL,US,BETHESDA, MD, vol. 8, 1 November 1997 (1997-11-01), page 85A XP002062360 ISSN: 1059-1524 the whole document	8-11
A	YANG Z ET AL: "SOLUBLE CD14 AND LIPOPOLYSACCHARIDE-BINDING PROTEIN FROM BOVINE SERUM ENABLE BACTERIAL LIPOPOLYSACCHARIDE-MEDIATED CYTOTOXICITY AND ACTIVATION OF BOVINE VASCULAR ENDOTHELIAL CELLS IN VITRO" JOURNAL OF LEUKOCYTE BIOLOGY,US,FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL, vol. 59, no. 2, 1 February 1996 (1996-02-01), pages 241-247, XP002062361 ISSN: 0741-5400	12
X	SETOGUCHI M ET AL: "MOUSE AND HUMAN CD14 (MYELOID CELL-SPECIFIC LEUCINE-RICH GLYCOPROTEIN) PRIMARY STRUCTURE DEDUCED FROM CDNA CLONES" BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION,NL,ELSEVIER, AMSTERDAM, vol. 1008, 1 January 1989 (1989-01-01), pages 213-222, XP002062356 ISSN: 0167-4781 the whole document	1-5,7
A	IKEDA A ET AL: "MOLECULAR CLONING OF BOVINE CD14 GENE" JOURNAL OF VETERINARY MEDICAL SCIENCE - NIHON JUIGAKU ZASSHI,JP,JAPANESE SOCIETY OF VETERINARY SCIENCE, TOKYO, vol. 59, no. 8, 1 January 1997 (1997-01-01), pages 715-719, XP002062359 ISSN: 0916-7250	1-4
X	STELTER F. ET AL.: "The myeloid differentiation antigen CD14 is N- and O-glycosylated. Contribution of N-linked glycosylation to different soluble CD14 isoforms" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, no. 2, March 1996 (1996-03), pages 457-464, XP000905218 the whole document	1-5,7,10

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
[redacted]EP 99/07911

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9961468	A	02-12-1999	AU	4025899 A	13-12-1999
WO 9822580	A	28-05-1998	AU	5045198 A	10-06-1998
			CN	1238010 A	08-12-1999
			CZ	9901751 A	17-11-1999
			EP	0941322 A	15-09-1999
			PL	333413 A	06-12-1999
WO 9204908	A	02-04-1992	DE	4029227 A	19-03-1992
			AT	141510 T	15-09-1996
			CA	2072626 A	15-03-1992
			DE	59108104 D	26-09-1996
			EP	0500844 A	02-09-1992
			JP	5502893 T	20-05-1993

by fax and post

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

LOCK, Graham J.
FRY HEATH & SPENCE
The Old College
53 High Street
Horley, Surrey RH6 7BN
GRANDE BRETAGNE

RECEIVED

FAX NO: 1293776837

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	08.02.01
Applicant's or agent's file reference: P58091L/GJL/jrm	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/07911	15/10/1999	20/10/1998

Applicant
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel.+49 89 2399-8090

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
NO 5650/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/07911	15/10/1999	20/10/1998
International Patent Classification (IPC) or national classification and IPC		
C07K14/705 ...		
Applicant		
SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 11 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).

   These annexes consist of a total of 3 sheets.

CORRECTED VERSION

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/05/2000	08.02.01
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Wimmer, G Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/07911

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-24	as originally filed				

Claims, No.:

1-19	as received on	07/11/2000	with letter of	06/11/2000	

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	12, 14, 16-19
	No:	Claims	1-11, 13, 15
Inventive step (IS)	Yes:	Claims	16-19
	No:	Claims	12, 14

Industrial applicability (IA) Yes: Claims 1-15
No: Claims

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item IV

Unity of invention.

The present patent application lacks unity of invention as required by Art. 34.3 PCT.

In order to comply with the requirements for unity of invention, it is necessary that a single general inventive concept is present (Rule 13.1 PCT).
The claims 1-15 of the present application relate to a CD14 variant isolated from mature human milk. Claims 16-19, however, refer to the use of any CD14 variant in the treatment or prevention of a GI tract disorder.

The feature common to all of these claims would therefore be any CD14 variant. This, however, is not novel, and does therefore not constitute a "special technical feature", i.e., does not define a contribution over the prior art (Rule 13.2 PCT).

Therefore, two groups of inventions are present:

I) Claims 1-15, relating to a CD14 variant isolated from mature human milk;

II) claims 16-19, relating to the use of CD14 in GI tract disorder.

Since, however, the examination of both groups poses no excessive effort, no invitation to restrict or to pay additional fees is extended at the moment.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability.

The application does not meet the requirements of Art. 33 PCT since claims 1-11, 13 and 15 are not novel, and **claims 12 and 14 do not appear to contain an inventive step.**

1) Reference is made to the following documents (the document numbering corresponds to their order of citation in the international search report):

D2: WO 98 22580 A (ALIZADEH KHIAVI K ;FILIPP DOMINIK (CA); JULIUS

MICHAEL H (CA); WEL) 28 May 1998 (1998-05-28)

D4: WANG Y ET AL: 'DETECTION AND IDENTIFICATION OF SOLUBLE CD14 IN BOVINE MILK' MOLECULAR BIOLOGY OF THE CELL,US,BETHESDA, MD, vol. 8, 1 November 1997 (1997-11-01), page 85A XP002062360 ISSN: 1059-1524

D6: SETOGUCHI M ET AL: 'MOUSE AND HUMAN CD14 (MYELOID CELL-SPECIFIC LEUCINE-RICH GLYCOPROTEIN) PRIMARY STRUCTURE DEDUCED FROM CDNA CLONES' BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION,NL,ELSEVIER, AMSTERDAM, vol. 1008, 1 January 1989 (1989-01-01), pages 213-222, XP002062356 ISSN: 0167-4781

D8: STELTER F. ET AL.: 'The myeloid differentiation antigen CD14 is N- and O-glycosylated. Contribution of N-linked glycosylation to different soluble CD14 isoforms' EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, no. 2, March 1996 (1996-03), pages 457-464, XP000905218

Novelty under Art. 33(2) PCT.

3) The application describes the isolation of a CD14 protein from mature milk. The applicants describe this protein to have an amino acid sequence which is substantially identical to that of known serum CD14, but argue that it is different by certain features:

a) no O-glycosylation
b) different molecular weight as determined by SDS-page
c) protein is not detected by MY4 antibody in western blot.

It appears to the IPEA that these criteria do not sufficiently distinguish the protein of the application from already known CD14 variants.

ad a) For the CD14 variants disclosed in the prior art, there is no indication of O-glycosylation to be present.

For instance, document D2 describes the isolation of a bovine CD14. The applicants discuss this disclosure on pg. 3 of the description, and speculate that the protein disclosed in D2 is different to the subject of the invention of the current application. However, the authors of D2 solely indicate three possible *N*- glycosylation sites of the protein described (pg. 19, lines 9-12; pg. 26, lines 16-18), and no O-glycosylation.
D6 discloses the cloning of human and murine CD14. It is discussed that human CD14 shows 4 N-glycosylation sites, but O-glycosylation was not assessed.

ad b) Different gel mobility can not be sufficiently shown. For instance, Figure 1/3A of the application shows a comparison of human serum CD14 and the protein of the application. From the gel resolution, it appears that the protein of the application has the same gel mobility as the human serum CD14 alpha band. Applicants specifically describe the protein in question to be 48 kDa in size; serum CD14 variants have been described to have 48 kDa (D6, human serum CD14 alpha form), 48-55 kDa (D4, bovine CD14 isolated from milk)

ad c) The fact that the protein in question is not detected by the MY4 antibody appears dubious.
Firstly, the experiments (Test2, Test4) raise doubts, since the MY4 antibody there was successfully used to block the action of milk CD14, indicating that MY4 does not only react, but also neutralize the CD14 variant active in milk.

Moreover, the applicants do not describe the western blotting conditions under which this result was achieved. It does moreover not emanate clearly from the description of the Figures if the MY4 antibody was used in the

western blots shown in Fig. 1/3B (just an "Anti-C-terminus" antibody is mentioned), however arguments later submitted by the applicants support this interpretation.
If the antibody used in fig. 1/3B is in fact MY4, it appears that the absence of a band for the protein in question (lanes 1-3) coincides with the absence of the alpha band of serum CD14. Consistent with point 3b above, this strengthens the suspicion that the protein described by the applicants would in fact correspond to the alpha form of serum CD14.

Consequently, the protein in question is not distinguishable from known CD14 variants, since a) O-glycosylation has not been shown for known CD14 variants, b) the molecular mass of the protein in question appears to be the same as that of several CD14 variants known in the art, and c) since the alleged non-reactivity with MY-4 antibody appears to be contradictive, and cannot be taken to define subject-matter.

Moreover, document D8 already describes enzymatic treatment of CD14 isoforms to remove N- and O-glycosylation. For this reason alone, human serum CD14 isoforms, which are not o-glycosylated, are anticipated by the prior art.

Therefore, the protein cannot be regarded as being novel.

The bovine protein of D2 would, accordingly, be novelty destroying for claims 1 and 3, and the protein of D4 for claims 2, 4, 5 (three N-glycosylation sites are discussed in D2) and 6 (alpha form of the protein). The deglycosylated protein of D8 as well anticipates subject-matter of claims 1-6.
Consequently, claims 1-6 do not comply with Art. 33(2) PCT.

4) Since there is no indication that the protein of e.g. D2 could not also be isolated from mature bovine milk, rather than from bovine colostral whey, claim 7 can also not be considered to be novel.

5) A method according to claim 8 is disclosed e.g. in D4, wherein soluble CD14 is extracted from bovine milk. Claim 8 is therefore not novel.

6) "A composition which comprises a protein according to claims 1-7" obviously includes most milk products, since the protein referred to is described to be present in milk. Since, moreover, "a physiologically acceptable carrier, adjuvant or diluent" may e.g. also be water, claims 9 - 11 are disclosed with e.g. any cheese (containing water, milk fat, and casein fractions). These claims therefore lack novelty.

7) Document D2 already states the incorporation of CD14 in infant formula (pg. 5, lines 12-14). Claims 13 and 15 are therefore not novel.

8) The defined use of CD14 for the treatment of a gastro-intestinal tract disorder has, however, not been described in the prior art. Claims 16-19 are therefore novel.

Inventive Step under Art. 33(3) PCT.

9) Even though the protein of the application cannot be distinguished from the proteins of the prior art through the feature stated in the application, that it carries "no O- glycosylation", no inventive step can be seen in the provision of such a protein.

As described above, D8 already discloses such proteins. Furthermore, it is standard procedure in the art to express any given coding sequence (including human serum CD14) in yeast and bacteria. In this, the proteins produced will differ from the same proteins expressed in mammalian cells, through their glycosylation pattern.

10) Dependent claims 12 and 14 do not contain any features which would make these claims novel and inventive.

11) Although the protein in question is *per se* not novel, the applicants find that there are differences of this isoform vs. certain seral isoforms, in their binding behaviour to MEM-18 monoclonal antibody, and of stimulating intestinal epithelial cells.

Therefore, the use of said CD14 isoform in the treatment of gastro-intestinal tract disorders, can be viewed to contain an inventive step.

Consequently, an inventive step is acknowledged for claims 16-19.

Industrial Applicability under Art. 33(4) PCT.

12) For the assessment of the present claims 16-19 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI

Certain published documents (Rule 70.10 PCT).

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99 61468 A	02/12/1999	27/05/1999	27/05/1998

Re Item VII

Certain defects in the application.

Figure 1/3 lacks sufficient description. The vague description given on pg. 6 does not explain lane PS, nor, most importantly, the antibody used (and detection conditions). It is especially unclear whether the antibody used in panel B, lanes 1-3, is in fact the MY4 antibody. Furthermore, the apparent difference between lanes 1-3 and 5-8 needs to be explained. Lane 4 is not labelled.

Re It m VIII

Certain observations and clarity.

Claims 1-3 refer to proteins of defined homology of amino acid sequence to human serum CD14, and bovine or buffalo CD14, respectively.
However, since no sequence information has been included in the application, these features do not clearly define subject-matter, since the prior art contains several similar, albeit different, versions of human serum CD14 sequences (source: Swissprot and Genbank databases).
Examination of the claims herein was based on the reference to WO98/22580 given in the application.

CLAIMS

1. An isolated protein having no O-glycosylation and at least 70% homology of amino acid sequence with human serum CD14.

2. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of human serum CD14.

3. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of bovine or buffalo CD14.

4. A protein according to any preceding claim wherein the protein has a plurality of N-glycosylation sites.

5. A protein according to claim 4 which comprises from about 3 to about 5 N-glycosylation sites.

6. A protein according to any preceding claim wherein the presence of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

7. A protein according to any preceding claim isolated from mature human, bovine or buffalo milk.

8. A method of production of a protein according to any preceding claim which comprises isolating it from mature milk.

9. A composition which comprises a protein according to any one of claims 1 to 7 excluding mature milk.

10. A composition according to claim 9 which comprises a physiologically

acceptable carrier, adjuvant or diluent.

11. A composition according to claim 9 or 10 which comprises a casein fraction and milk fat.

12. A composition according to any one of claims 9 to 11 which comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal permeability increasing factor (BPI) or a mixture thereof.

13. A composition according to any one of claims 9 to 12 in the form of an infant formula or enteral composition.

14. A composition according to any one of claims 9 to 13 which comprises at least 25μg/ml of a protein according to any one of claims 1 to 7.

15. A method of production of a composition according to any one of claims 9 to 14 which comprises adding a protein according to any one of claims 1 to 7.

16. Use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

17. Use according to claim 16 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Crone's disease, ulcerative colitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

18. A method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which

retains the bioactivity of CD14.

19. A method of treatment according to claim 18 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Crone's disease, ulcerative colitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

Seen CM 18/8/00. RV

Stefania, please could you pass on as necessary. Thanks.

PCT

WRITTEN OPINION (PCT Rule 66)

To:
Lock, Graham
55, Avenue Nestlé
CH-1800 Vevey
SUISSE

18 AOUT 2000

Date of mailing (day/month/year)	14.08.2000
REPLY DUE	**within 3 month(s)** from the above date of mailing
Applicant's or agent's file reference	NO 5650/WO
International application No.	PCT/EP99/07911
International filing date (day/month/year)	15/10/1999
Priority date (day/month/year)	20/10/1998
International Patent Classification (IPC) or both national classification and IPC	C07K14/705
Applicant	SOCIETE DES PRODUITS NESTLE S.A. et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain document cited
VII	☒	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 20/02/2001.

Name and mailing address of the international preliminary examining authority:
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Authorized officer / Examiner
Wimmer, G

Formalities officer (incl. extension of time limits)
Vullo, C
Telephone No. +49 89 2399 8061

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-24 as originally filed

Claims, No.:

1-19 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees, the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with for the following reasons

and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees:

see separate sheet

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-11, 13
Inventive step (IS)	Claims	12, 14-19
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item IV
Unity of invention.

The present patent application lacks unity of invention as required by Art. 34.3 PCT.

In order to comply with the requirements for unity of invention, it is necessary that a single general inventive concept is present (Rule 13.1 PCT).
The claims 1-15 of the present application relate to a CD14 variant isolated from mature human milk. Claims 16-19, however, refer to the use of *any* CD14 variant in the treatment or prevention of a GI tract disorder.

The feature common to all of these claims would therefore be *any* CD14 variant. This, however, is not novel, and does therefore not constitute a "special technical feature", i.e., does not define a contribution over the prior art (Rule 13.2 PCT).

Therefore, two groups of invetions are present:
I) Claims 1-15, relating to a CD14 variant isolated from mature human milk;
II) claims 16-19, relating to the use of CD14 in GI tract disorder.

Since, however, the examination of both groups poses no excessive effort, no invitation to restrict or to pay additional fees is extended at the moment.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability.

The application does not meet the requirements of Art. 33 PCT since **claims 1-11 and 13 are not novel**, and **claims 12 and 14-19 do not appear to contain an inventive step**.

1) Reference is made to the following documents (the document numbering corresponds to their order of citation in the international search report):

D2: WO 98 22580 A (ALIZADEH KHIAVI K ;FILIPP DOMINIK (CA); JULIUS MICHAEL H (CA); WEL) 28 May 1998 (1998-05-28)

D4: WANG Y ET AL: 'DETECTION AND IDENTIFICATION OF SOLUBLE CD14 IN BOVINE MILK' MOLECULAR BIOLOGY OF THE CELL,US,BETHESDA, MD, vol. 8, 1 November 1997 (1997-11-01), page 85A XP002062360 ISSN: 1059-1524

D6: SETOGUCHI M ET AL: 'MOUSE AND HUMAN CD14 (MYELOID CELL-SPECIFIC LEUCINE-RICH GLYCOPROTEIN) PRIMARY STRUCTURE DEDUCED FROM CDNA CLONES' BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION,NL,ELSEVIER, AMSTERDAM, vol. 1008, 1 January 1989 (1989-01-01), pages 213-222, XP002062356 ISSN: 0167-4781

D8: STELTER F. ET AL.: 'The myeloid differentiation antigen CD14 is N- and O-glycosylated. Contribution of N-linked glycosylation to different soluble CD14 isoforms' EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, no. 2, March 1996 (1996-03), pages 457-464, XP000905218

Novelty.

2) The subject-matter of claim 1 includes proteins of at least 70% homology to human serum CD14, which have no O-glycosylation.

First, the applicants provide no amino acid sequence for the claimed protein. It is therefore not possible to assess levels of homology to human serum CD14. Therefore, the claims my only be directed to the protein disclosed in the application, i.e., the protein which can be isolated by the means described, from mature milk.

3) The applicants claim that their protein is different to the ones already disclosed by certain features:
a) no O-glycosylation
b) different molecular weight as determined by SDS-page
c) protein is not detected by MY4 antibody in western blot.

It appears to the IPEA that these criteria do not sufficiently distinguish the protein of the application from already known CD14 variants.

ad a) For the CD14 variants disclosed in the prior art, there is no indication of O-glycosylation to be present.

For instance, document D2 describes the isolation of a bovine CD14, which is 73.1% homologous to human serum CD14. The applicants discuss this disclosure on pg. 3 of the description, and speculate that the protein disclosed in D2 is different to the subject of the invention of the current application. However, the authors of D2 solely indicate three possible N-glycosylation sites of the protein described (pg. 19, lines 9-12; pg. 26, lines 16-18).
D6 discloses the cloning of human and murine CD14. It is discussed that human CD14 shows 4 N-glycosylation sites, but O-glycosylation was not assessed.

ad b) Different gel mobility can not be sufficiently shown. For instance, Figure 1/3A of the application supposedly shows a comparison of human serum CD14 and the protein of the application. Although the Figure is insufficiently described (see sect. VII), the IPEA assumes that lanes 1-5 show different concentrations of the protein in question, and lane 6 ("NP") is human serum CD14. from the gel resolution, it appears that the protein of the application has the same gel mobility as the human serum CD14 alpha band.

ad c) The fact that the protein in question is not detected by the MY4 antibody appears dubious.
Firstly, the applicants do not describe the western blotting conditions under which this result was achieved. It does moreover not emanate from the description of the Figures if the MY4 antibody was used in the western blots shown in Fig. 1/3B (just an "Anti-C-terminus" antibody is mentioned). If, however, the antibody used in fig. 1/3B is in fact MY4, and that lanes 1-3 should express the absence of a band for the protein of the application, but the presence with serum CD14, it appears that also the alpha band of "NP" disappeared with this antibody, consistent with point 3b above.
Finally, the experiments (Test2, Test4) raise doubts, since the MY4 antibody there was successfully used to block the action of milk CD14, indicating that MY4 does not only react, but also neutralize the CD14 variant active in milk.

For the above reasons, and as a distinction of the protein in question from others on the basis of amino acid comparison is not possible, since according sequences have not been provided, the protein cannot be regarded as being novel.

The bovine protein of D2 would, accordingly, be novelty destroying for claims 1 and 3, and the protein od D4 for claims 2, 4, 5 (three N-glycosylation sites are discussed in D2) and 6 (alpha form of the protein).
Consequently, **claims 1-6** do not comply with Art. 33(2) PCT.

4) Since there is no indication that the protein of e.g. D2 could not also be isolated from mature bovine milk, rather than from bovine colostral whey, **claim 7** can also not be considered to be novel.

5) Although it is not clear, to which protein **claim 8** is referring to (see sect. VIII), it is assumed that "A method of production of a protein according to any of the preceding claims" is intended by the applicants.
Such a method is disclosed e.g. in D4, rendering the claim not novel.

6) "A composition which comprises a protein according to claims 1-7 excluding mature milk" obviously includes *any* milk product which is not mature milk. Since "a physiologically acceptable diluent" may e.g. also be water, **claims 9 - 11** have been extensively disclosed.

7) Document D2 already states the use of the protein in infant formula (pg. 5, lines 12-14). **Claim 13** is therefore not novel.

Inventive Step.

8) Even though the protein of the application cannot be distinguished from the proteins of the prior art through the feature stated in the application, that it carries "no O-glycosylation", no inventive step can be seen in the provision of such a protein, if presence of O-glycosylation for the CD14 variants of the prior art could be assured.

Document D8, for instance, describes that the level of N-glycosylation has no effect on the behaviour of human serum CD14 to bind LPS (lipopolysaccharides, endotoxins; see pg. 463, right column, lines 7-12). Although the influence of O-glycosylation has not been assessed in D8, it appears that N-glycosylation has no effect on the binding behaviour of CD14 to LPS.
There is no indication in the application that a surprising effect is achieved by the absence of O-glycosylation. The applicants solely describe properties of the protein in question, which are present or can be assumed to be present with other CD14 variants, such as the bovine CD14 of D2.

9) Dependent **claims 12, 14 and 15** do not contain any features which would make these claims novel and inventive.

10)The use of a CD14 variant in the treatment or prevention of a Gastrointestinal Tract disorder cannot be viewed as involving an inventive step. Since the reactivity of CD14 with endotoxins of gram-negative bacteria is commonly known in the art, and since gram-negative bacteria are predominantly found in the large intestine, such an application is obvious.
Claims 16-19 are therefore not regarded as containing an inventive step.

Re Item VI
Certain published documents (Rule 70.10 PCT).

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99 61468 A	02/12/1999	27/05/1999	**27/05/1998**

Re Item VII
Certain defects in the application.

1) The application does not provide an amino acid sequence for the protein in question. Consequently, although the protein itself may be claimed, since it might be isolated through the process described, no levels of homology to other proteins can be assessed.
Therefore, all claims referring to proteins with certain homology percentages lack support by the description (Art. 6 PCT).

2) Figure 1/3 lacks sufficient description. The vague description given on pg. 6 does not explain lane PS, nor, most importantly, the antibody used (and detection conditions). It is especially unclear whether the antibody used in panel B, lanes 1-3, is in fact the MY4 antibody. Furthermore, the apparent difference between lanes 1-3 and 5-8 needs to be explained. Lane 4 is not labelled.

Re Item VIII

Certain observations and clarity.

Claim 8 refers to "A method of production of *the protein*". Likewise, claim 15 refers to "A method of production of *the composition*". It should be stated which protein or composition is referred to.

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Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PROTEIN FOR TREATMENT OR PREVENTION OF A GI TRACT DISORDER

(57) Abstract

A new isolated protein is described having no O–glyclosylation and at least 70 % homology of amino acid sequence with human serum CD14. In addition, a composition comprising an effective amount of the protein and use of a CD14 variant in the treatment or prevention of a disorder of the gastro–intestinal tract of a mammal are described. In particular the disorder is selected from the group which comprises inflammatory bowel disease, chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

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Protein For Treatment Or Prevention of A GI Tract Disorder

The present invention relates to a new isolated protein from mature human milk; a composition comprising it; a method for manufacture of the protein or the composition; use of the protein, a variant or fragment thereof in the manufacture of a medicament or nutritional product for the treatment or a gastro-intestinal (hereinafter GI) tract disorder; and a method of treatment or prevention of the disorder which comprises administering an effective amount of the protein or composition..

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only".

Within the context of this specification, the terms AMP, CMP, GMP and UMP are taken to mean respectively the monophosphates of adenosine, cytidine, guanosine and uridine and their nucleotide equivalents which include polymeric RNA, ribo-nucleosides, ribonucleoside containing adducts and di-and triphosphate ribo-nucleotides.

It is well known that infant formulae are generally designed to resemble human milk as closely as possible. However, a plurality of constituents in human milk are bioactive and, because of synergies among the constituents, the inclusion of just one or a few of them in the infant formula may not produce the bioactivity observed in human milk.

In addition, bioactivity of the constituents may be affected by heat treatment for sterilisation and long term storage of the formula.

These problems are compounded in view of the fact that not all of the constituents have been identified and there are variations in the concentration of components which are present, possibly due to variations of mother's diets. Therefore there are difficulties in formulating infant formulae which resemble human milk.

It is known that pharmaceutical compounds have wide application and some may be used in the treatment of patients suffering from disorders of the GI tract. However, a number of the known compounds are not naturally occurring and in view of this, patients may be reluctant to be administered them. In the light of this there is a need for the provision of new products which include naturally occurring compounds that have a nutritional or therapeutic effect.

A problem with some commercially available products is that they give rise to side effects such as nausea, bloating, cramping, allergy etc. Clearly there is a need for a product which does not give rise to these side effects.

The method of administration of a nutritional or therapeutic compound is an important consideration. Intravenous or subcutaneous administration requires expertise and compared to oral administration it is not as safe, convenient or acceptable to the patient. In the light of these concerns it is clear that there is a need for new nutritional or therapeutic products which can be administered orally.

The protein, CD14, is a myeloid cell-surface glycoprotein which acts as a receptor for bacterial lipopolysaccharide. It is well documented that monocyte/ macrophage activation by lipopolysaccharides via membrane CD14 (mCD14) triggers the release of a variety of pro-inflammatory, immunoregulatory and cytotoxic molecules such as TNF-α, IL-1, IL-6, IL-8, oxygen radical products and nitric oxide. mCD14 lacks transmembrane and cytoplasmic domains. It is anchored to the cell membrane by a glycosyl-phosphatidylinositol linkage.

In addition to the membrane bound form, soluble CD14 (sCD14) has been identified in normal human blood serum, hereinafter referred to as serum sCD14. Serum sCD14 exists in two forms, serum sCD14α (49 kDa) and serum sCD14β (55 kDa). It has been demonstrated that serum sCD14 binds lipopolysaccharides and mediates the lipopolysaccharide–induced activation of cells that lack mCD14, including epithelial and endothelial cells and astrocytes, as well as mCD14 expressing cells, such as monocytes and neutrophils.

The main source of serum sCD14 in normal human plasma is the monocyte. Monocytes release the two isoforms of serum sCD14, α and β, into plasma. The

former is produced by limited proteolysis from membrane-bound CD14 and the latter is directly derived from the intracellular compartment. The sCD14β:sCD14α ratio in culture supernatant of normal monocytes is approximately 2:1. However, in plasma from normal donors the serum sCD14α levels are either similar to, or even higher than, those of serum sCD14β, suggesting that the amount of serum sCD14β released *in vivo* is either lower or other cell types may contribute to the plasma pool.

A substantial concentration of serum sCD14 is found in normal human plasma, 2-3 μg/ml. In sera of septic patients global concentrations of serum sCD14 are elevated, reaching around 4 μg/ml. It has been reported a correlation between high levels of serum sCD14 at the onset of septic shock and poor outcome in septic patients.

WO98/22580 discloses the presence of a protein in bovine colostral why which has some amino acid sequence similarity with human serum sCD14. It is speculated that this protein could be the bovine variant of CD14. Figure 7 of the document shows the differences between the amino acid sequences of bovine colostral whey CD14, and the sequences of human serum CD14 and mouse serum CD14 which were known in the literature. In addition, the document describes affinity purification of a CD14 protein from human colostrum using a sepharose column having the monoclonal antibody 63D3 bound to it. However, the document does not describe an isolated CD14 variant purified from mature milk. Instead, colostrum is disclosed as the source of the protein. This is early milk from about the first one or two days post partum. It is yellow in comparison to the whiter colour of mature milk, it comprises a higher lipid content than mature milk and has a different nutritional value. It is well known that the mammary gland of first few days post partum undergoes a process of "physiological inflammation" and a large number of inflammatory cells such as neutrophils are present. This process allows leakage of serum factors including serum CD14 into the colostrum. Crucially, in view of this, the disclosure of WO98/22580 is considered to disclose purification of serum sCD14 or a complex mixture of proteins including serum CD14 from colostrum.

Remarkably, a new variant of CD14 has now been identified. It has been isolated from mature human milk. Suprisingly, this new variant is not the same

as serum CD14. Indeed, as described below, it differs from the known CD14 variants including the variants disclosed by WO98/22580 at least insofar as the new protein comprises no O- glycosylation. In stark contrast, the known CD14 variants including human serum sCD14 have both O- and N- glycosylation. Furthermore, it has now been found that CD14, a variant or fragment thereof retaining the bioactivity of CD14, or a composition comprising it is effective in the treatment of GI tract disorders.

Accordingly, in a first aspect, the invention provides an isolated protein having no O-glyclosylation and at least 70% homology of amino acid sequence with human serum CD14.

In a second aspect, the invention provides a method of production of the protein which comprises isolating it from mature milk.

In a third aspect, the invention provides a composition which comprises the protein excluding mature milk.

In a forth aspect, the invention provides a method of production of the composition which comprises adding an embodiment of the protein according to the invention.

In a fifth aspect the invention provides use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

In a sixth aspect the invention provides a method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which retains the bioactivity of CD14.

The new protein has the unique capacity of being capable of mediating bacterial interaction with intestinal surfaces. Furthermore, if the new variant is included in an infant formula, the formula closely resembles mature human milk in its protective capacity of the intestinal surface.

Preferably, the amino acid sequence of an embodiment of the protein is at least about 90% homologous with the amino acid sequence of human serum CD14, more preferably at least about 95% homologous, even more preferably it is substantially identical. In alternative embodiments the amino acid sequence preferably has these degrees of homology with the amino acid sequence of bovine or buffalo CD14.

Preferably, an embodiment of the protein has at least one N- glyclosylation site. Preferably it has a plurality of N- glycosylation sites, more preferably from about 1 to about 10. Even more preferably, an embodiment of the protein has from about 3 to about 5 N- glyclosylation sites, most preferably 4.

Preferably, the presence of an embodiment of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

Preferably an embodiment of the protein is isolated from mature milk. More preferably it is isolated from mature human, bovine or buffalo milk. Most preferably the protein is isolated mature milk sCD14 (hereinafter referred to as mmsCD14). In alternative embodiments it is produced recombinantly by standard techniques.

Preferably an embodiment of the composition comprises an embodiment of the protein together with a physiologically acceptable carrier, adjuvant or diluent. More preferably it comprises a compound extracted from milk, even more preferably a plurality of compounds extracted from milk. Most preferably the composition is an infant formula or enteral composition.

Preferably an embodiment of the composition comprises a casein fraction and milk fat. These two components of milk based products provide the advantage that they can preserve molecules from the proteolytic activity of the digestive tract. The biological activity of the protein according to the invention takes place in the small intestine after the passage through the gastric environment.

Preferably an embodiment of the composition comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal

permeability increasing factor (BPI) or a mixture thereof. The advantage provided by these molecules with a protein according to the invention is that they participate in molecular recognition of the bacterial products and/or complement its defensive function.

The invention will now be described with reference to the accompanying drawings in which:

Figure 1A illustrates a comparative SDS-PAGE pattern of sCD14 of mature human milk (several dilutions (from 1:6 to 1:100) and in normal human plasma serum (NP, 1:50), with a rabbit polyclonal antibody.

Figure 1B illustrates the lack of milk CD14 detection by an antibody specific for the C-terminus peptide of the β serum CD14 molecule.

Figure 2 shows interleukine-8 release by undifferentiated HT29 cells following a 24h incubation with 100 ng/ml of *E. Coli* LPS in the presence of either 10% human serum (HS, pooled serum AB^+) or 1.7% human milk (HM, pooled mature human milk). To verify the role of sCD14 in induction, 20 μg/ml of monoclonal anti-CD14 antibody (MY4) or isotype control (IgG2b) was added to serum or milk before stimulation with LPS.

Figure 3 shows interleukine-8 release by undifferentiated HT29 cells following a 24h incubation with 2.5×10^6 *E. Coli* in the presence of either 10% human serum (HS, pooled serum AB^+) or 1.7% mature human milk (HM, pooled breast milk). The role of sCD14 was confirmed by the inhibition of the IL-8 production when 20 μg/ml of monoclonal anti-CD14 antibody (MY4) was added to serum or milk before stimulation with LPS.

This invention is based upon the finding that mature milk comprises an unknown variant of CD14. The new molecule has a high homology at the amino acid sequence with the serum sCD14 molecule, however it has a different gel mobility that either of the two serum soluble forms, is the secretory product of the mammary gland epithelial cell, has a different glycosylation pattern with regard to the known forms, and has a unique capacity for mediating bacterial interaction with intestinal surfaces.

Furthermore, the invention relates to the use of the new form of soluble CD14 molecule, present in human, bovine and buffalo milk, in infant formula, clinical nutrition and animal feed.

In contrast with serum sCD14, where the α and β forms have been described, in human milk only a single major species can be detected. It differs in its tissue of origin -it is produced by the mammary gland epithelial cells- and the electrophoretic mobility.

Human serum and milk samples were analysed by SDS-PAGE under reducing conditions (Phastsystem®, Pharmacia) followed by western blotting with rabbit polyclonal anti CD14 antibody (Ab) and detection by enhanced chemiluminescence method (Amersham). The anti-CD14 monoclonal Ab, MY4, extensively used in this field for the detection of membrane-bound and soluble serum CD14 –both α and β- , failed to detect sCD14 in human milk.

The amino acid sequence including the N- and C-terminus ends of the mature human milk derived molecule is substantially identical to that of serum CD14 showing that milk and serum CD14 are highly homologous.

N- and C-terminal sequence analysis shows that milk CD14 is not posttranslationally truncated and corresponds therefore to the β form found in serum. But, whereas the β-form has now been found to migrate on SDS-PAGE with about 55KDa, milk CD14 migrates on SDS-PAGE only with about 48 KDa. This difference indicates that mature human milk and serum CD14 have different glycosylation patterns. Indeed, whereas CD14 has been reported to be N- and O-glycosylated, deglycosylation assays with milk CD14 showed no O-glycosylation. The presence of only N-glycosylation was confirmed by LC-MS analysis of N-glycosidase F treated mmsCD14 in that the resulting molecular mass corresponded to the theoretical molecular mass based on the published amino acid sequence. The main form of mature human milk sCD14 is therefore different from blood sCD14 that was reported to be N- and O-glycosylated.

An optical biosensor assay (Iasys, Affinity Sensors, Cambridge, UK) which implements advanced resonant mirror optical biosensor technology was utilised

to study the specific interaction of human serum CD14 and mature human milk CD14 with the mAb MEM-18 (Biogenesis, Poole, UK).

The MEM-18 mAb and a control isotype antibody (mouse IgG1, BectonDickinson) were immobilised in 10 mM Na-acetate at pH 5 on a dual well carboxymethyl dextran matrix of an IAsys cuvette using standard EDC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-HCl] and NHS (*N*-hydroxysuccinimide) coupling chemistry and subsequent blocking with ethanolamine. Running buffer was PBS/Tween 20.

Human serum (diluted 1:5 in PBS/Tween 20) and mature human milk (diluted 1:30 in PBS/Tween 20) samples (5 µL) were pipetted into the cuvette wells and their respective binding to both the specific MEM-18 and control surface were logged with time. It was observed that the mature human milk sample yielded an approximate 10-fold decrease in biosensor response within the first 2-3 minutes of protein-antibody interaction compared to that of the human serum sample. This indicates that the milk-borne CD14 has different/slower binding kinetics to MEM-18 than the serum-derived CD14.

The effect of milk-borne sCD14 on LPS stimulation of the mucosal surface, specifically the epithelial layer, was studied with IEC models *in vitro*. More specifically the IEC production of the cytokines IL-8 and TNF-α following endotoxin challenge has now been analysed. Three different human IEC lines, HT-29, SW620 and Caco-2 were used.

It is known that lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide binding protein and serum sCD14. LPS induces IL-8 secretion by IEC in the presence of human serum. This effect is maximum at an LPS concentration of 100 ng/ml. mmsCD14 has now been tested for its capacity to mediate LPS effects on IEC. Different mature human milk concentrations were assayed in IEC serum-free culture media containing a fixed dose of LPS (100 ng/ml). IL- 8 production increased with increasing concentrations of human milk (0-10%).

The contribution of mmsCD14 to stimulation of HT-29 cells by gram-negative non-pathogenic *Escherichia coli* has now been tested. An increasing quantity of

IL-8 secretion has been observed with increasing concentrations of bacteria in the range of 1 $X10^3$, to 5 $X10^6$ CFU per ml in the presence of 2% human milk. Furthermore, stimulation with 10^6 E. coli/ml of cell culture in the presence of 2% human milk induced ENA-78. Furthermore stimulation of intestinal epithelial cells with LPS and E. coli in the presence of milk-derived sCD14 induced expression of the β-defensin , HBD-2, that participates in the protection of mucosal surfaces against bacteria. Furthermore, mmsCD14 is more efficient than serum sCD14 in its stimulatory function of intestinal epithelial cells by bacteria or lipopolysaccharide.

An embodiment of the enteral composition of the invention preferably comprises an effective amount of mmsCD14, and preferably contains at least about 25 μg/ml of mmsCD14. The other components of the enteral composition are those conventionally added to infant formulae or enteral products and may be at least one of those described below.

Preferably the mmsCD14 may be any form of mmsCD14, but is preferably the form found in mature human milk and recognised by a polyclonal rabbit antibody. This form has a molecular mass of approximately 48 KDa, an electrophoretic mobility faster than the serum alpha form and cannot be recognised by the commercially available anti-CD14 antibody MY4. Alternatively the mmsCD14 may be extracted from bovine, buffalo, goat or sheep milk. In addition, it can be produced by recombinant microorganisms; for example recombinant fungi or yeast.

The supplementation of baby formula with this mmsCD14 has physiological benefit during the baby's neonatal period. This period is characterised by bacterial challenge due to bacterial colonisation and antigen challenge that could lead to inflammation, septic conditions or an allergic reaction.

The invention includes the use of a CD14 variant or fragment thereof having CD14 activity of mediating interaction of bacteria with an intestinal surface in the manufacture of a composition for the treatment or prevention of a disorder of the gastro-intestinal tract of a mammal. In particular the disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic

hepatitis, necrotising enterocolitis, neonotal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body. Mammals most likely to develop these disorders are premature, mal-nourished, immuno-depressed or mammals under trauma conditions.

Preferably the CD14 variant or fragment thereof is an embodiment of the protein according to the invention.

An embodiment of a composition according to the invention preferably comprises about 1.8 to about 4.5 g protein/100 kcal, preferably about 1.8 to about 3.6 g/100 kcal. The protein may be any suitable protein such as cow's milk protein, casein, whey, soy protein, egg protein, pea protein or a mixture thereof. The protein may be in the form of a salt, such as a caseinate. It may be an isolate or concentrate. Furthermore, the protein may be in intact or hydrolysed form. Alternatively or in addition, free amino acids may be used. Preferably a mixture of the whey : casein mass ratio is 60:40.

Whey protein can be prepared to have reduced allergenicity using conventional techniques such as described in U.S. Patent 5039532. For example, it can be prepared by electrodialysis or ultrafiltration.

An embodiment of a composition according to the invention preferably comprises about 7g to about 14g/100kcal of carbohydrate or provide 40 to about 60% of calories as carbohydrate. The carbohydrate can be supplied in a conventional form including simple form or complex form. Simple carbohydrates include lactose, maltose, sucrose and corn syrup solids. Complex carbohydrates include starches and maltodextrins. Starch may be precooked or pregelitanised.

An embodiment of a composition according to the invention preferably comprises about 3.3 to 6.5 g/100 kcal of fat. The fat can be supplied in a suitable form including saturated fat, monounsurated fat (MUFA), polyunsaturated fat (PUFA) or a mixture thereof. Preferably, fat is provided as about 1/3 saturated fat, about 1/3 MUFA and about 1/3 PUFA. Saturated fat includes butyric, valeric, caproic, capylic, decanoic, lauric, myristic, palmitic, steraic, arachidic, behenic and lignoceric fatty acids. MUFAs include palmitoleic, oleic, elaidic, vaccenic

and erucic fatty acids. Preferred PUFAs are C18, C20 or C22 ω-3 or C18, C20 or C22 ω-6 polyunsaturated fatty acids. Most preferred are the C20 or the C22 ω-3 or C20 or C22 ω-6 polyunsaturated fatty acids. These include not only arachidonic acid (ARA) but also eicosapentaenoic acid (EPA) and docoshexaenoic acid (DHA).

More than one PUFA can be added. In this case, two or more PUFAs may be from a different source, they can be added either separately or together during preparation of the composition according to the invention. For example, fish oil containing DHA may be mixed with one or more microbial oils containing another PUFA (e.g. ARA).

An embodiment of a composition according to the invention preferably comprises a carbohydrate : lipid weight ratio which is greater than 60:40. Preferably the ratio of carbohydrate to lipid is between 65:35 and 90:10. More preferably still it is between 70:30 and 85:15.

An embodiment of a composition according to the invention preferably comprises a nutritionally acceptable quantity of a minerals or vitamin selected from the group which comprises calcium, phosphorus, potassium, sodium, chloride, magnesium, iron, copper, zinc, manganese, iodine, selenium, vitamin A, vitamin D, vitamin E, vitamin K1, vitamin B1, vitamin B2, vitamin B6, vitamin B12, vitamin C, pantothenic acid, niacin, folic acid, biotin, choline, inositol or a mixture thereof.

An embodiment of a composition according to the invention preferably comprises an additional nutritional factor selected from taurine, carnitine, etc. The composition may contain at least about 7.5 mmoles carnitine /100 kcal.

An embodiment of a composition according to the invention preferably comprises one or more of AMP, CMP, GMP or UMP.

An embodiment of a composition according to the invention preferably comprises a pharmaceutically acceptable filler, color additive, taste modifier, non-antagonistic antibiotic, pharmaceutical, medicament or mixture thereof.

An embodiment of a composition according to the invention is preferably in a form suitable for ingestion orally or for administration by enteral tube feeding. More preferably it is an oral formulation.

A composition of the present invention is preferably formulated to provide about 600 to about 800 kcals/litre, more preferably about 670 kcals/litre. The total volume per dose is about 50 top about 200 ml preferably about 100 to about 150 ml.

The composition is preferably a solid, in which case it is preferably dried, and optimally in the form of a powder. Preferably, it is miscible or dispersible in an aqueous liquid, such as water.

Alternatively the composition is a liquid which is ready for use, or a concentrated liquid which can be diluted before use, preferably with water.

An embodiment of a composition according to the invention is preferably prepared by a method which comprises the following steps; (1) Standardising pasteurised milk (skimmed, evaporated or whole milk) by the addition of whey protein concentrate, minerals, water-soluble vitamins, trace elements and carbohydrates at a high temperature, for example 60°C, (2) mixing vegetable oil, oil-soluble emulsifier, at least one oil-soluble vitamin and antioxidant at high temperature, for example 60°C, (3) adding the oil mixture obtained from (2) to the standardised milk obtained from (1) with sufficient agitation to allow mixing, (4) homogenising the mixture obtained in (3) in two stages at high temperature and pressure, for example 60°C at 150 and then 30 bar, (5) cooling the emulsion obtained under (4) to a low temperature, for example 5°C, (6) adding water-soluble vitamins, minerals and trace elements to the cooled emulsion if desired, (7) sterilising the emulsion obtained under (6) on-line at ultra high temperature (UHT) and/or in an appropriate container to obtain a formula in the form of a sterile liquid or pasteurising and spray drying the emulsion (6) to give a spray dried powder which is filled into appropriate containers and (8), if desired, adding other dry ingredients, e.g. vitamins, minerals, trace elements, whey protein concentrate and carbohydrates to the spray dried powder by dry mixing.

The enteral composition may be supplemented with an embodiment of the protein according to the invention by adding it to the oil during processing. This provides the advantage that a production plant, or the process need not be significantly modified. However, adding the protein at such an early stage can have disadvantages because it may be degraded. In the light of this, alternatively it is added at a later stage. This provides the advantage that exposure of the protein to unfavourable conditions is minimised. Preferably, the protein is added after drying.

The protein can be added in a variety of forms. It may be added in solution, for example in a lipid and/or an oil composition. The oil may contain solely the protein or it may contain a number of other ingredients. If a solid composition is used, the protein may be encapsulated in capsules or it maybe in a powdered form.

In an embodiment of a process according to invention it is preferred that the starting oil phase does not contain any PUFAs. Preferably they are added later, preferably after drying.

The enteral composition and the use of this enteral composition according to the invention are described in further detail in the tests and examples described below where percentages are given by weight, except where otherwise indicated.

Test 1: Ion exchange chromatography for purification of mmsCD14 from mature milk (bovine, buffalo, goat or sheep) and supernatant of CD14 cDNA transfected cells.

The purification of mmsCD14 from milk or culture supernatant of transfectant cells was performed by ion exchange chromatography. Diluted milk samples or conditioned medium of CD14 cDNA transfectant cells were applied to a Mono Q10/10 column equilibrated with 20mM ethanolamine pH 9.5 . After washing, the column was subjected to a linear gradient of NaCl (0-500mM) in the equilibrating buffer. Fractions are collected and the mmsCD14 content was determined by ELISA.

Further characterisation by reverse phase HPLC was carried out to confirm the purity of the protein. The selected fractions were pooled , desalted with PBS, and kept aliquoted at –70°C until further use. The purified material was routinely characterised by N-terminal sequencing, amino acid analysis, SDS-Page, and mass spectrometry.

Test 2: Detection of mmsCD14.

In vitro stimulation studies of intestinal epithelial cell lines (HT29 and SW620) with several dose of LPS (Sigma O55B5) or with *E. Coli* bacteria in the presence and/or absence of either mature human, buffalo or bovine milk, and anti-CD14 antibodies were carried out.

The human intestinal epithelial cells HT29 (CD14-negative) were stimulated with varying concentrations of LPS (0.1 to 1000ng/ml) in the presence or absence of human AB serum (10%). After 24h culture at 37°C, culture supernatants were collected and tested for IL-8 release by ELISA. The levels of IL-8 were compared with those detected by stimulating the HT29 with the same amounts of LPS in the absence of serum and the presence of human breast milk (1.7%). In some experiments, the anti-CD14 specific mAb MY4 (20 µg/ml), which binds to the same epitope as LPS on the sCD14 molecule, was used to block the stimulation mediated by the human milk or serum.

As shown in figure 2, neither LPS alone nor human serum or human milk alone were able to induce significant levels of IL-8. However LPS was capable of inducing substantial amounts of IL-8 in the presence of either human serum or human milk. This effect was abrogated by CD14-specific monoclonal antibodies MY4, but not by an irrelevant monoclonal antibody (IgG2b, MOPC-141). Similar results were obtained with the other intestinal epithelial cell line SW620 and with differenciated HT29 cells.

Test 3: mmsCD14 quantitative determination in human milk

Concentration of mmsCD14 in human milk was determined by a human CD14-specific ELISA test (IBL, Germany). Samples taken in the first days of lactation had a higher amount of sCD14 than the overall average: 75.4± 19.1µg/ml

compared to 52.9 ± 24.0μg/ml. This is thought to be at least partially due to leakage of serum sCD14 into the colostrum.

Test 4: Functional effect of milk-derived sCD14

In vitro stimulation studies of astrocytoma (U373) cell lines with several dose of lipopolysaccharides, 10pg/ml to 5pg/ml, in the presence and/or absence of human or bovine milk, human or foetal calf serum, and anti-CD14 antibodies were carried out.

The human astrocytoma cell line U373 (CD14 negative) was stimulated with varying concentrations of lipopolysaccharides, 1pg/ml to 10ng/ml, in the presence or absence of human AB serum (10% or 1%) and soluble recombinant human CD14 (3 ug/ml). After 48h culture at 37°C, culture supernatants were collected and tested for IL-6 release by ELISA. The levels of IL-6 were compared with those detected by stimulating the U373 with the same amounts of lipopolysaccharides in the absence of serum and the presence of 1% human milk. In some experiments, the anti-CD14 specific mAbs MEM-18 and MY4 (15ug/ml), which bind to the same epitope as lipopolysaccharides on the sCD14 molecule, was used to block stimulation mediated by human milk or serum.

Neither lipopolysaccharides alone nor 0.5% or 1% human milk alone are able to induce significant levels of IL-6. However, lipopolysaccharides were capable of inducing substantial amounts of IL-6 in the presence of mature human milk (0.5 and 1%). This effect was abrogated by two CD14-specific monoclonal, antibodies , MEM-18 and MY4, but not by an irrelevant monoclonal antibody (iMab MOPC-21). MEM-18 and MY4 were also able to block the release of IL-6 induced by lipopolysaccharides in the presence of 10 AB human serum, albeit partially, suggesting that other mechanisms of IL-6 release independent of CD14 may operate in human serum but not in mature milk.

In conclusion, mature milk-derived sCD14 is biologically active and capable of mediating cell activation by lipopolysaccharides. Differences in the biological activities are observed between serum sCD14 and mature milk-derived sCD14.

In addition, the contribution of mmsCD14 on the stimulation of intestinal epithelial cells by Gram-negative non-pathogenic bacteria was tested. HT29 cells were stimulated with varying amounts of *E. Coli* (1×10^3 to 5×10^6 CFU per ml) in the presence or absence mature human breast milk (1.7%). After 24h culture at 37°C, culture supernatants were collected and tested for IL-8 release by ELISA. The levels of IL-8 were compared with those detected by stimulating the HT29 with the same amounts of LPS in the absence of milk and the presence of human AB serum (10%).

As shown in figure 3, *E. Coli* alone was unable to induce significant levels of IL-8. However Incubation of E.Coli in the presence of either human serum or human milk was capable of inducing substantial amounts of IL-8. This effect was abrogated by CD14-specific monoclonal antibodies MY4, but not by the irrelevant antibody (IgG2b).

Test 5: Production of mmsCD14 from natural sources.

Purification of mmsCD14 with anti-sCD14 monoclonal antibodies, production of anti-mmsCD14 polyclonal antibody and large scale purification immunoaffiniity chromatography and ion exchange chromatography.

a. Immunoaffinity chromatography for purification of sCD14 from mature milk (bovine, buffalo, goat or sheep)

Small scale purification of mmsCD14 was performed by immunoaffinity chromatography using a purified anti-sCD14 monoclonal antibody, MY4 (Coulter Immunotech, USA). Briefly, a diluted mature milk sample was applied to an anti-CD14-Sepharose 4B matrix. After washing, the column was eluted with 100 mM Glycine.HCl, pH 2.5. Fractions are collected and neutralized. The mmsCD14 content of each fraction was determined by an anti-sCD14 ELISA test (IBL, Hamburg, Germany), and the purity was analysed by SDS-PAGE and silver staining. The selected fractions were pooled and kept in –20°C until further use.

Polyclonal antibodies against mmsCD14 were obtained by immunisation of rabbits with the antigen-adjuvant mixture. Briefly, an emulsion composed of 0.5

mg/ml purified mmsCD14 and 2 ml complete Freund adjuvant (Sigma, St Louis, MO) with insoluble Mycobacterium tuberculosis bacilli was injected into multiple intamuscular sites. The animal was bled 14 days following the first immunisation. Booster immunisation using incomplete Freund adjuvant (Sigma) was performed 6 weeks after priming immunisation and with intervals of 2-3 weeks thereafter. Bleedings were performed 10-14 days after immunisation. The antibody titers were performed by ELISA.

Small scale purification of sCD14 from milk was performed by immunoaffinity chromatography using the purified polyclonal antiserum.

Example 1

Formula for low-birth-weight infants, in powder form was prepared.

The formula has the composition (per 100 g of powder) which is described in the table I below.

Table I

[ALPREM NA071]

Nutrient	**Unit**	**Amount**
Total fat	g	24
Total protein	g	14.4
Total carbohydrates	g	55.9
mmsCD14	mg	20
Sodium	mg	180
Potassium	mg	530
Chloride	mg	280
Calcium	mg	490
Phosphorus	mg	320
Magnesium	mg	54
Manganese	µg	34
Vitamin A	IU	1500
Vitamin D	IU	490
Vitamin E	IU	9.8

Vitamin K_1	µg	59
Vitamin C	mg	79
Vitamin B_1	mg	0.29
Vitamin B_2	mg	0.66
Niacin	mg	4.9
Vitamin B_6	mg	0.37
Folic acid	µg	290
Pantothenic acid	mg	2.2
Vitamin B_{12}	µg	1.1
Biotin	µg	11
Choline	mg	37
Nutrient	**Unit**	**Amount**
Inositol	mg	22
Taurine	mg	39
Carnitine	mg	7.9
Iron	mg	7.4
Iodine	µg	49
Copper	mg	0.44
Zinc	mg	3.7

The formula was reconstituted by mixing 142g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can vary to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 2

Starter formula for infants (from birth to 4-5 months), in powder form was prepared.

The formula has the composition (per 100 g of powder) which is described in the table II below.

Table II

Nutrient	Unit	Amount
Total fat	g	25.8
Total protein	g	11.5
Total carbohydrates	g	57.8
mmsCD14	mg	20
Sodium	mg	120
Potassium	mg	460
Chloride	mg	360
Calcium	mg	320
Nutrient	**Unit**	**Amount**
Phosphorus	mg	160
Magnesium	mg	35
Manganese	µg	36
Vitamin A	IU	1500
Vitamin D	IU	310
Vitamin E	IU	6.1
Vitamin K_1	µg	42
Vitamin C	mg	41
Vitamin B_1	mg	0.31
Vitamin B_2	mg	0.69
Niacin	mg	3.8
Vitamin B_6	mg	0.38
Folic acid	µg	46
Pantothenic acid	mg	2.3
Vitamin B_{12}	µg	1.1
Biotin	µg	11
Choline	mg	38
Inositol	mg	23
Taurine	mg	41
Carnitine	mg	8.2
Iron	mg	6.1
Iodine	µg	25
Copper	mg	0.31

Zinc	mg	3.8

The formula was reconstituted by mixing 132g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can vary to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 3

A formula for infants, from 5 months of age, is prepared.

The formula has the composition (per liter of ready to use preparation) which is described in the table III below.

Table III

Nutrient	**Unit**	**Amount**
Total fat	g	29.4
Total protein	g	22.4
Total carbohydrates	g	78.9
mmsCD14	mg	25
Sodium	mg	320
Potassium	mg	1060
Chloride	mg	760
Phosphorus	mg	680
Calcium	mg	820
Magnesium	mg	73
Manganese	µg	41
Vitamin A	IU	2700
Vitamin D	IU	600
Vitamin E	IU	8
Vitamin K_1	µg	30
Vitamin C	mg	67

Vitamin B_1	mg	1
Vitamin B_2	mg	1.6
Niacin	mg	18
Vitamin B_6	mg	1.3
Folic acid	µg	200
Pantothenic acid	mg	4.7
Vitamin B_{12}	µg	1.3
Biotin	µg	23
Choline	mg	67
Nutrient	**Unit**	**Amount**
Inositol	mg	34
Iron	mg	11
Iodine	µg	140
Copper	mg	0.8
Zinc	mg	8

The composition given above can be varied to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 4

A formula for infants, from 5 months of age, containing partly hydrolyzed protein for low allergenicity, in powder form was prepared. The formula has the composition (per 100g of powder) which is described in the followed table IV.

Table IV

Nutrient	**Unit**	**Amount**
Total fat	g	22
Total protein	g	15.2
Total carbohydrates	g	57
mmsCD14	Mg	20

Sodium	Mg	170
Potassium	Mg	580
Chloride	Mg	340
Calcium	Mg	540
Phosphorus	Mg	230
Magnesium	Mg	39
Manganese	µg	29
Vitamin A	IU	1900
Vitamin D	IU	440
Vitamin E	IU	5.8
Nutrient	**Unit**	**Amount**
Vitamin K_1	µg	22
Vitamin C	Mg	49
Vitamin B_1	Mg	0.73
Vitamin B_2	Mg	1.2
Niacin	Mg	13
Vitamin B_6	Mg	0.97
Folic acid	µg	150
Pantothenic acid	Mg	3.4
Vitamin B_{12}	µg	0.97
Biotin	µg	17
Choline	Mg	49
Inositol	Mg	24
Iron	Mg	8.3
Iodine	µg	100
Copper	Mg	0.58
Zinc	Mg	5.8

The formula was reconstituted by mixing 138g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can be varied to accommodate for local directives concerning the amounts of specific ingredients permitted. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 5

A formula that can be administered to an infant suffering from bovine milk allergy, comprising ultrafiltered/microfiltered extensively hydrolyzed protein, in powder form was prepared.

The formula had the composition (per 100 g of powder) which is described in the following table V.

Table V

Nutrient	**Unit**	**Amount**
Total fat	g	24
Total protein	g	16.5
Total carbohydrates	g	52
mmsCD14	mg	20
Sodium	mg	290
Potassium	mg	600
Chloride	mg	500
Calcium	mg	400
Phosphorus	mg	250
Magnesium	mg	60
Manganese	µg	337
Vitamin A	IU	1200
Vitamin D	IU	290
Vitamin E	IU	5.8
Vitamin K_1	µg	26
Vitamin C	mg	39
Vitamin B_1	mg	0.29
Vitamin B_2	mg	0.63
Niacin	mg	3.6
Vitamin B_6	mg	0.34
Folic acid	µg	43
Pantothenic acid	mg	2.2
Vitamin B_{12}	µg	0.96

Biotin	µg	11
Choline	mg	58
Inositol	mg	29
Taurine	Mg	39
Carnitine	Mg	14
Iron	Mg	7.2
Iodine	µg	39
Copper	Mg	0.39
Zinc	Mg	3.4
Nutrient	**Unit**	**Amount**
Chromium	µg	14
Molybdenum	µg	39
Fluoride	µg	140

The formula was reconstituted by mixing 150g of powder to 900ml of water to give 1l of a ready-to-drink preparation. The composition can be varied to accommodate local directives concerning the amounts of specific ingredients permitted. Other trace elements (e.g. selenium) may be added in an adequate amount according to the age of the intended consumer. Nucleosides and/or nucleotides can also be present.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

Claims

1. An isolated protein having no O-glyclosylation and at least 70% homology of amino acid sequence with human serum CD14.

2. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of human serum CD14.

3. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of bovine or buffalo CD14.

4. A protein according to any preceding claim wherein the protein has a plurality of N- glycosylation sites.

5. A protein according to claim 4 which comprises from about 3 to about 5 N- glyclosylation sites.

6. A protein according to any preceding claim wherein the presence of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

7. A protein according to any preceding claim isolated from mature human, bovine or buffalo milk.

8. A method of production of the protein which comprises isolating it from mature milk.

9. A composition which comprises a protein according to any one of claims 1 to 7 excluding mature milk.

10. A composition according to claim 9 which comprises a physiologically acceptable carrier, adjuvant or diluent.

11. A composition according to claim 9 or 10 which comprises a casein fraction and milk fat.

12. A composition according to any one of claims 9 to 11 which comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal permeability increasing factor (BPI) or a mixture thereof.

13. A composition according to any one of claims 9 to 12 in the form of an infant formula or enteral composition.

14. A composition according to any one of claims 9 to 13 which comprises at least 25μg/ml of a protein according to any one of claims 1 to 7.

15. A method of production of the composition which comprises adding a protein according to any one of claims 1 to 7.

16. Use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

17. Use according to claim 16 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

18. A method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which retains the bioactivity of CD14.

19. A method of treatment according to claim 18 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis,

infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

Figure 1

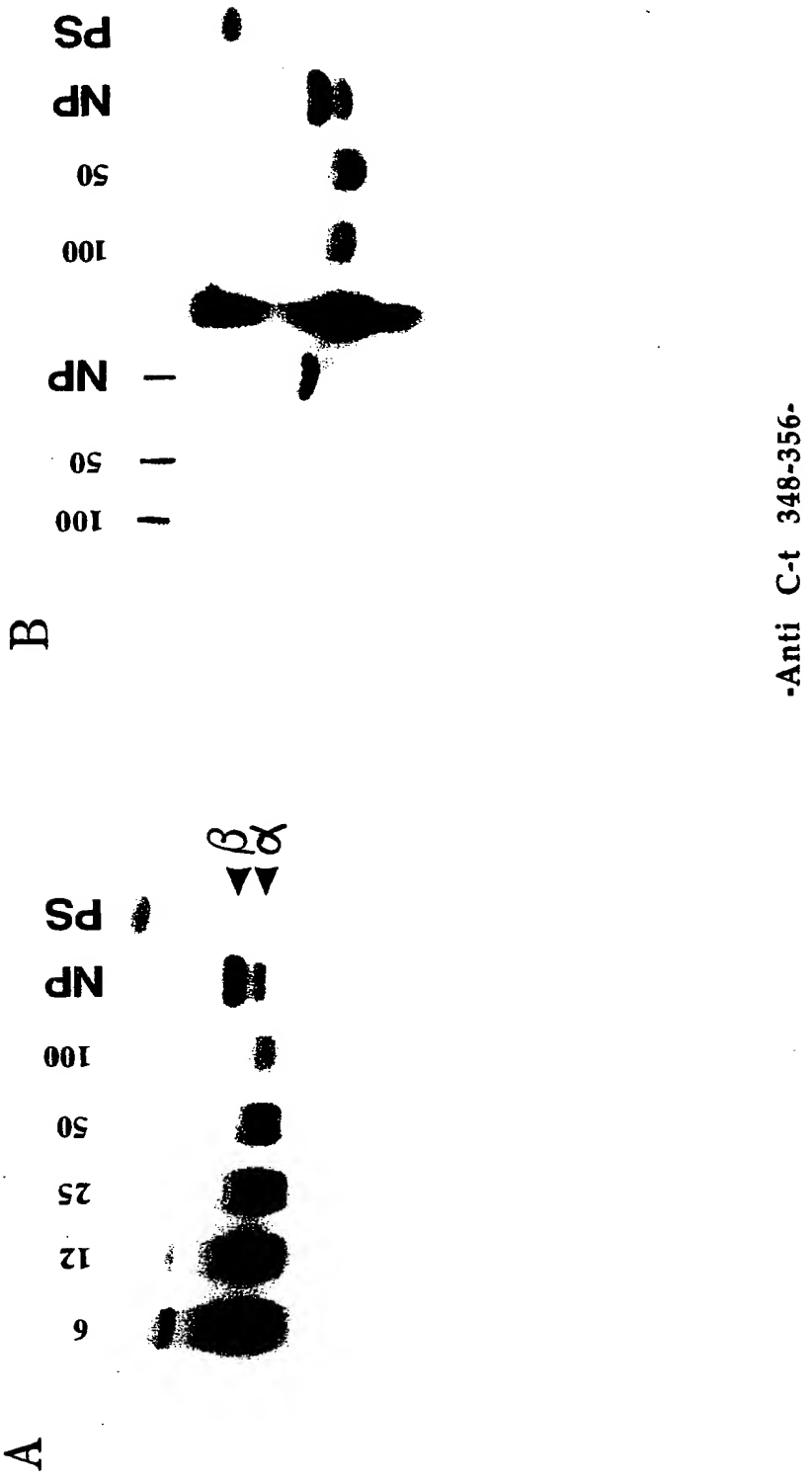


Figure 2

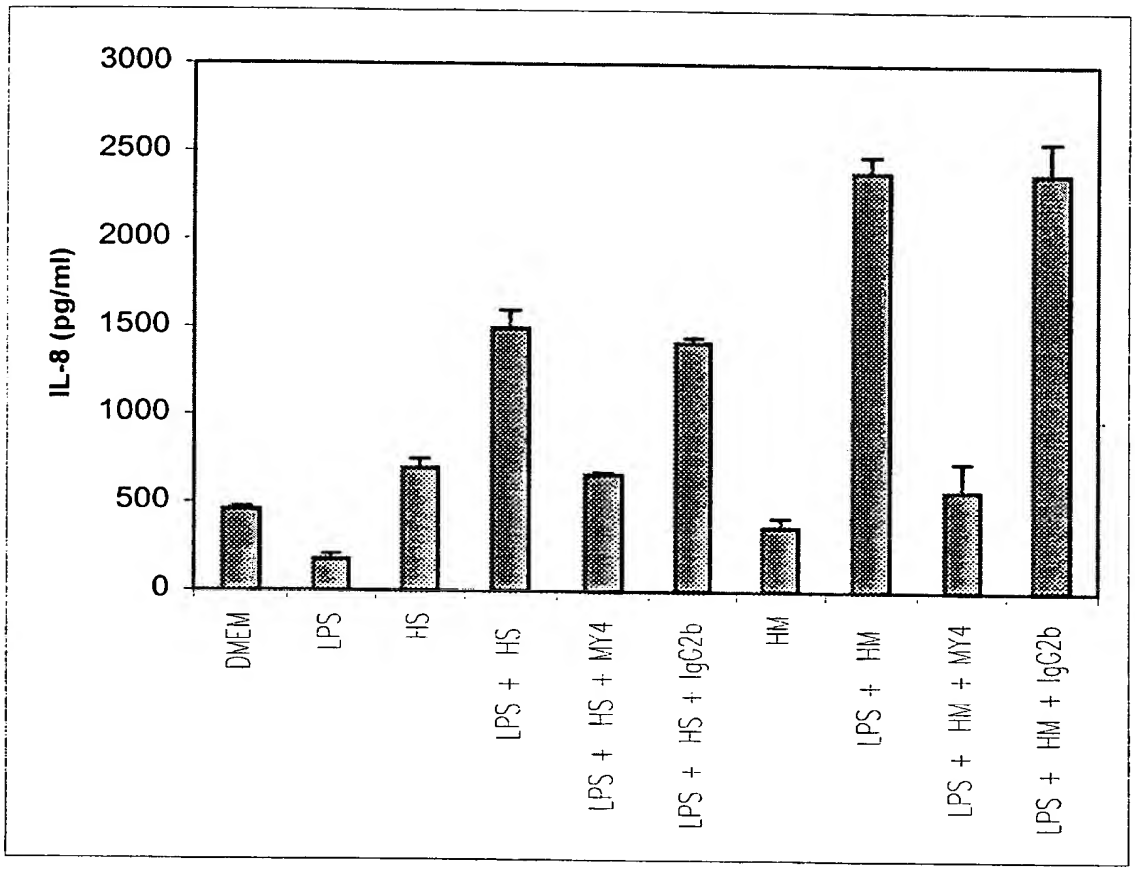
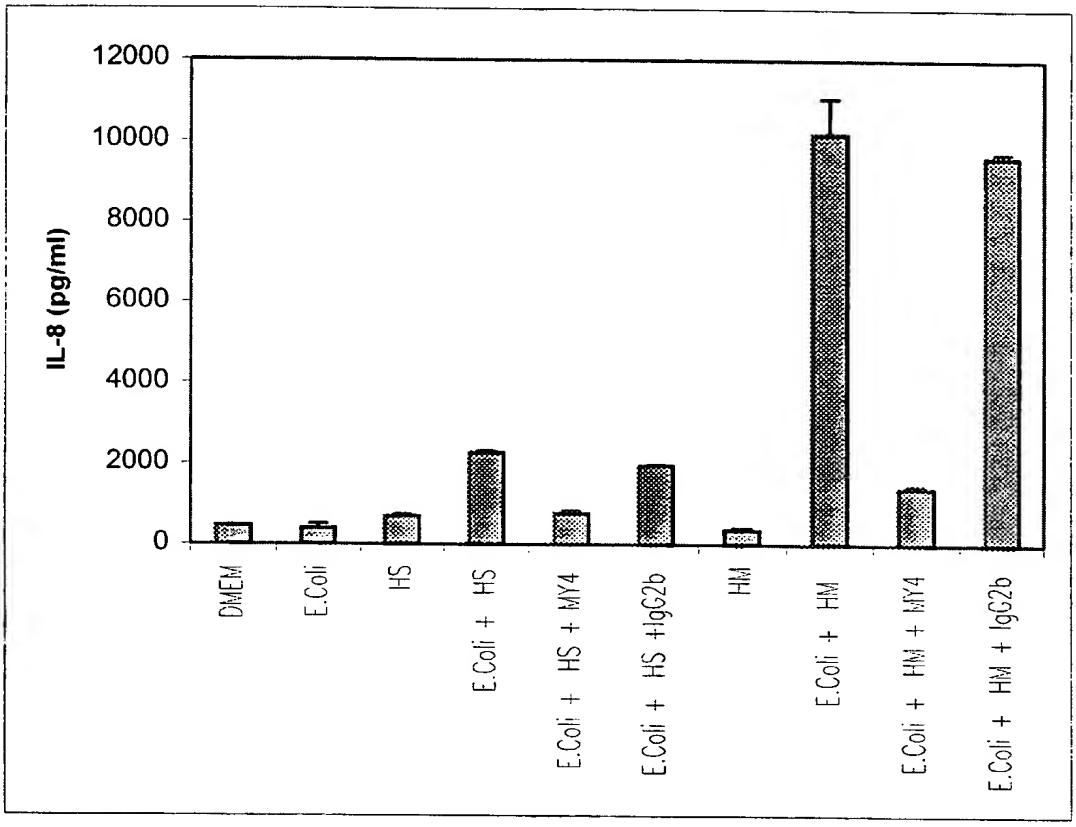

IL-8 (pg/ml)
3000
2500
2000
1500
1000
500
0
DMEM
LPS
HS
LPS + HS
LPS + HS + MY4
LPS + HS + IgG2b
HM
LPS + HM
LPS + HM + MY4
LPS + HM + IgG2b

Figure 3


IL-8 (pg/ml)
12000
10000
8000
6000
4000
2000
0
DMEM
E.Coli
HS
E.Coli + HS
E.Coli + HS + MY4
E.Coli + HS +IgG2b
HM
E.Coli + HM
E.Coli + HM + MY4
E.Coli + HM + IgG2b

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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27 July 2000 (27.07.00)

(54) Title: PROTEIN FOR TREATMENT OR PREVENTION OF A GASTROINTESTINAL TRACT DISORDER

(57) Abstract

A new isolated protein is described having no O–glyclosylation and at least 70 % homology of amino acid sequence with human serum CD14. In addition, a composition comprising an effective amount of the protein and use of a CD14 variant in the treatment or prevention of a disorder of the gastro–intestinal tract of a mammal are described. In particular the disorder is selected from the group which comprises inflammatory bowel disease, chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

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INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/EP 99/07911

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 A23L1/305 A23J1/20 A61P1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A23L A23J A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 99 61468 A (GEMMA BIOTECHNOLOGY LTD ;FILIPP DOMINIK (CA); JULIUS MICHAEL H (CA) 2 December 1999 (1999-12-02) the whole document	1-5,7-19
X	WO 98 22580 A (ALIZADEH KHIAVI K ;FILIPP DOMINIK (CA); JULIUS MICHAEL H (CA); WEL) 28 May 1998 (1998-05-28) the whole document	1-5, 7-10, 12-15,18
X	WO 92 04908 A (IMTOX PRIVATINSTITUT FUER IMMU) 2 April 1992 (1992-04-02) the whole document	8-10,13, 14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2000	09/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG Y ET AL: "DETECTION AND IDENTIFICATION OF SOLUBLE CD14 IN BOVINE MILK" MOLECULAR BIOLOGY OF THE CELL,US,BETHESDA, MD, vol. 8, 1 November 1997 (1997-11-01), page 85A XP002062360 ISSN: 1059-1524 the whole document	8-11
A	YANG Z ET AL: "SOLUBLE CD14 AND LIPOPOLYSACCHARIDE-BINDING PROTEIN FROM BOVINE SERUM ENABLE BACTERIAL LIPOPOLYSACCHARIDE-MEDIATED CYTOTOXICITY AND ACTIVATION OF BOVINE VASCULAR ENDOTHELIAL CELLS IN VITRO" JOURNAL OF LEUKOCYTE BIOLOGY,US,FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL, vol. 59, no. 2, 1 February 1996 (1996-02-01), pages 241-247, XP002062361 ISSN: 0741-5400	12
X	SETOGUCHI M ET AL: "MOUSE AND HUMAN CD14 (MYELOID CELL-SPECIFIC LEUCINE-RICH GLYCOPROTEIN) PRIMARY STRUCTURE DEDUCED FROM CDNA CLONES" BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION,NL,ELSEVIER, AMSTERDAM, vol. 1008, 1 January 1989 (1989-01-01), pages 213-222, XP002062356 ISSN: 0167-4781 the whole document	1-5,7
A	IKEDA A ET AL: "MOLECULAR CLONING OF BOVINE CD14 GENE" JOURNAL OF VETERINARY MEDICAL SCIENCE - NIHON JUIGAKU ZASSHI,JP,JAPANESE SOCIETY OF VETERINARY SCIENCE, TOKYO, vol. 59, no. 8, 1 January 1997 (1997-01-01), pages 715-719, XP002062359 ISSN: 0916-7250	1-4
X	STELTER F. ET AL.: "The myeloid differentiation antigen CD14 is N- and O-glycosylated. Contribution of N-linked glycosylation to different soluble CD14 isoforms" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, no. 2, March 1996 (1996-03), pages 457-464, XP000905218 the whole document	1-5,7,10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/07911

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18 and 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No
PCT/EP 99/07911

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9961468 A	02-12-1999	AU 4025899 A	13-12-1999
WO 9822580 A	28-05-1998	AU 5045198 A	10-06-1998
		CN 1238010 A	08-12-1999
		CZ 9901751 A	17-11-1999
		EP 0941322 A	15-09-1999
		PL 333413 A	06-12-1999
WO 9204908 A	02-04-1992	DE 4029227 A	19-03-1992
		AT 141510 T	15-09-1996
		CA 2072626 A	15-03-1992
		DE 59108104 D	26-09-1996
		EP 0500844 A	02-09-1992
		JP 5502893 T	20-05-1993

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GERMANY

6 November 2000

Dear Sirs

International Patent Application No. PCT/EP99/07911
SOCIÉTÉ DES PRODUITS NESTLÉ S.A. *et al*
"Protein for Treatment or Prevention of a GI Tract Disorder"
Our Reference: P58091L/GJL/jrm

In reply to the written opinion of 14th August 2000 I am filing herewith in triplicate new claims 1-19 which replace claims 1-19 presently on file. Pages showing amendments made in manuscript are enclosed.

Novelty

The Examiner alleges that the application provides no amino acid sequence for the claimed protein. However, it is not in dispute that the amino acid sequence of serum CD14 was well known before the priority date. In the light of this, the known sequence was known to a person skilled in the art and should not need to be recited in the application. Furthermore, in case there was any doubt, the application refers to the publication WO98/22580 which gives this amino acid sequence.

The Examiner mentions in more than one place that an amino acid sequence comparison is not possible. However, it is not clear why the Examiner is bent on an amino acid sequence when the applicant is claiming a protein which is novel and inventive in the light of its glycosylation pattern (among other things). The applicant does not claim that the amino acid sequence differs from the known amino acid sequence of human serum CD14 - indeed, the applicant claims a protein having at least 70% homology with the known amino acid sequence.

The Examiner's objections stem from his unsupported and unacceptable allegation that "the applicants solely describe the properties of the protein in question, which

/....

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are present or can be assumed to be present" (see under paragraph 8). Indeed, the inventors who are of considerable skill in the art, have demonstrated that the novel protein claimed has a new O-glycosylation pattern, a different molecular weight and different MY4 binding properties compared to known CD14 proteins. The Examiner is not permitted to assume that the known CD14 variants are the same protein given these differences.

Evidence of the differences are that the new protein has a different electro phoretic mobility (page 7, lines 6-9); MY4 antibody failed to detect sCD14 in human milk (page 7, lines 11-16); the amino acid sequence of the mature human milk derived molecule is substantially identical to serum CD14 (page 7, lines 18-20); N and C terminal analysis shows that milk CD14 is not post-translationally truncated and corresponds to the CD14 ß form found in serum, but whereas the ß form migrates on SDS-PAGE with about 55 kDa, milk CD14 migrates on SDS-PAGE with only about 48 kDa (page 7, lines 22-27); N glycosylation has been confirmed by LC-MS analysis of N-glycosidase F treated mmsCD14 (page 7, lines 29-33); and de-glycosylation assays have revealed no O-glycosylation (page 7, lines 27-29).

It is not clear why the Examiner alleges that the protein derived from colostrum is the same as the protein derived from mature milk. He has no evidence that it is the same protein. In contrast, as mentioned above, the applicants have submitted evidence in the application which demonstrates that the claimed protein is not the same as known variants. Furthermore, the applicant submits that there is no suggestion in any of the cited documents which could lead a skilled person to the claimed invention.

With regard to the Examiner's comment at (4) that there is no indication that the protein of D2 could not be isolated from mature bovine milk, rather than bovine cholesterol whey, the applicants contend that the claimed protein having no O-glycosylation is only present in mature milk. Of course, it is well established that colostrum has a different composition to mature milk and different proteins present. Indeed, test 3 described in the application shows that there are differences in the CD14 composition in milk samples obtained in the first days of lactation compared to an overall average during lactation.

Furthermore, speculation with regard to whether or not the protein of D2 can be isolated from mature milk as well as colostrum is not relevant to whether a protein has been disclosed in the prior art having no O-glycosylation or whether a protein having the other claimed features has been disclosed including binding to the known anti-CD14 monoclonal antibody MY4.

For an invention to be anticipated, all features of the invention must be disclosed in a single published document. Indeed, the selection of no O-glycosylation from the various possibilities of O-glycosylation is a new invention in its own right. This feature is neither disclosed nor suggested in the cited documents and does not follow logically or plainly from them. /.....

With regard to the Examiner's allegation relating to novelty and inventive step of claim 8, the applicant submits that the claim is directed to a method of production of a novel method and inventive protein (see above). In the light of this, the claim relates to new and inventive subject matter. D4 does not disclose a method of production of the protein according to any one of claims 1-7.

With regard to the Examiner's allegations relating to claims 9-11, the applicant submits that the protein according to any one of claims 1-7 is new and inventive (see above); therefore, a composition comprising this protein is also new and inventive. In any event, the Examiner is invited to indicate where a protein having no O-glycosylation in composition with eg. water is "extensively disclosed". Furthermore, the Examiner is invited to indicate where a disclosure of the claimed protein with a casein fraction and milk fat can be found. The applicants submit that such a composition is novel and inventive in view of the fact that none of the cited documents either disclose such a composition or suggest anything which could lead to the invention without taking an inventive step.

With regard to claim 13, document D2 mentions use of a protein in infant formula. However, D2 does not mention use of the new and inventive protein according to any one of claims 1-7 in an infant formula. In the light of this, the claim relates to novel and inventive subject matter.

Inventive Step

The Examiner alleges that the claimed protein is not inventive in view of the fact that it has no O-glycosylation because according to D8 it has already been shown that N-glycosylation has no effect on binding behaviour.

In contrast to the Examiner's allegation, the applicant submits that the Examiner's arguments relating to N-glycosylation have no bearing on the importance of O-glycosylation. The fact that N-glycosylation may have little or no effect is irrelevant to the effect provided by O-glycosylation.

With regard to the Examiner's reference to a surprising effect achieved by the absence of O-glycosylation (part 8, second paragraph), the applicant submits that a skilled person would consider glycosylation in the same way that the Examiner has. A skilled person would assume, in the same way that the Examiner has, that because N-glycosylation makes little difference, O-glycosylation would also make little difference. However, remarkably, it has now been found that the claimed protein yields an approximate 10-fold decrease in biosensor response compared to serum derived CD14. A skilled person would not be able to predict this remarkable difference in activity without taking an inventive step. This surprising activity is discussed in the application on page 7, line 35, to page 8, line 18.

In addition, surprisingly, the claimed protein has been found to be more efficient than serum CD14 in its stimulatory function of intestinal epithelial cells by bacteria or lypopolysaccharide. This is discussed in the application at page 8, line 20, to page 8, line 9.

Furthermore, as mentioned by the Examiner, D2 and D4 disclose variants of CD14 with three and four glycosylation sites respectively and this would be expected to lead a skilled person to the conclusion that the degree of glycosylation is an important factor. Therefore, he would not be expected to jump from this to the invention of reducing O-glycosylation to zero.

With regard to the inventive step of the use and method of treatment claims (claims 16-19), the applicant alleges that "the reactivity of CD14 with endotoxins of gram-negative bacteria is commonly known in the art, and since gram-negative bacteria are predominantly found in the large intestine, such an application is obvious". However, the Examiner has not explained the basis of this allegation. In any event, there has been no suggestion that administration of the receptor can be carried out to combat GI tract infection. Indeed, a person skilled in the art before the priority date might have been expected to consider that administration of a bacterial receptor could lead to promoting infection. In contrast, surprisingly, the applicants have found that administration of the claimed protein reduces the risk of a GI tract disorder including inflammatory bowel disease, Crohne's disease, ulcerative colitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal micro flora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body. There is no suggestion in any of the cited documents that these conditions could be addressed by administration of the claimed protein.

Certain alleged defects

With regard to the need to provide an amino acid sequence, the Examiner is referred to the arguments presented in the first and second paragraphs under the sub-heading "Novelty".

With regard to the Examiner's allegations relating to an insufficient description of Figure 1, the applicant submits that there is sufficient information for a person skilled in the art to understand the parts of the Figure which relate to the claimed invention. As mentioned on page 6 of the application, Figure 1A illustrates a comparative SDS-page pattern of SCD14 of mature human milk. Several dilutions, from 1:6 to 1:100 are shown, together with the comparative pattern of normal human plasma serum (NP) at a dilution of 1:50 and the antibody used is a rabbit polyclonal antibody. In addition, Figure 1B illustrates the lack of milk CD14 detection by antibodies specific for the C-terminus peptide of the ß form of

serum CD14. This is described in additional detail on page 7, lines 11-16, where it is stated that the anti-CD14 monoclonal antibody, MY4, which is extensively used in this field for the detection of membrane bound and soluble serum CD14, failed to detect the CD14 in human milk.

Observations and Clarity

The Examiner's objections relating to the clarity of claims 8 and 15 have been addressed by claim amendments.

The Examiner is requested to take the applicant's amendments and arguments into account when drawing up the international preliminary examination report.

Yours faithfully

Lock, Graham James
Professional Representative
FRY HEATH & SPENCE

Encs.

Claims

1. An isolated protein having no O-glycosylation and at least 70% homology of amino acid sequence with human serum CD14.

2. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of human serum CD14.

3. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of bovine or buffalo CD14.

4. A protein according to any preceding claim wherein the protein has a plurality of N- glycosylation sites.

5. A protein according to claim 4 which comprises from about 3 to about 5 N- glycosylation sites.

6. A protein according to any preceding claim wherein the presence of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

7. A protein according to any preceding claim isolated from mature human, bovine or buffalo milk.

8. A method of production of ~~the~~ a protein according to any preceding claim which comprises isolating it from mature milk.

9. A composition which comprises a protein according to any one of claims 1 to 7 excluding mature milk.

10. A composition according to claim 9 which comprises a physiologically acceptable carrier, adjuvant or diluent.

11. A composition according to claim 9 or 10 which comprises a casein fraction and milk fat.

12. A composition according to any one of claims 9 to 11 which comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal permeability increasing factor (BPI) or a mixture thereof.

13. A composition according to any one of claims 9 to 12 in the form of an infant formula or enteral composition.

14. A composition according to any one of claims 9 to 13 which comprises at least 25μg/ml of a protein according to any one of claims 1 to 7.

15. A method of production of ~~the~~ a composition according to any one of claims 9 to 14 which comprises adding a protein according to any one of claims 1 to 7.

16. Use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

17. Use according to claim 16 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

18. A method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which retains the bioactivity of CD14.

19. A method of treatment according to claim 18 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis,

infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

CLAIMS

1. An isolated protein having no O-glycosylation and at least 70% homology of amino acid sequence with human serum CD14.

2. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of human serum CD14.

3. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of bovine or buffalo CD14.

4. A protein according to any preceding claim wherein the protein has a plurality of N-glycosylation sites.

5. A protein according to claim 4 which comprises from about 3 to about 5 N-glycosylation sites.

6. A protein according to any preceding claim wherein the presence of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

7. A protein according to any preceding claim isolated from mature human, bovine or buffalo milk.

8. A method of production of a protein according to any preceding claim which comprises isolating it from mature milk.

9. A composition which comprises a protein according to any one of claims 1 to 7 excluding mature milk.

10. A composition according to claim 9 which comprises a physiologically

acceptable carrier, adjuvant or diluent.

11. A composition according to claim 9 or 10 which comprises a casein fraction and milk fat.

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13. A composition according to any one of claims 9 to 12 in the form of an infant formula or enteral composition.

14. A composition according to any one of claims 9 to 13 which comprises at least 25μg/ml of a protein according to any one of claims 1 to 7.

15. A method of production of a composition according to any one of claims 9 to 14 which comprises adding a protein according to any one of claims 1 to 7.

16. Use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

17. Use according to claim 16 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Crone's disease, ulcerative colitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

18. A method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which

retains the bioactivity of CD14.

19. A method of treatment according to claim 18 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Crone's disease, ulcerative colitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.